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MECHANISMS OF OXYGEN TOXICITY AT THE CELLULAR LEVEL.(U)
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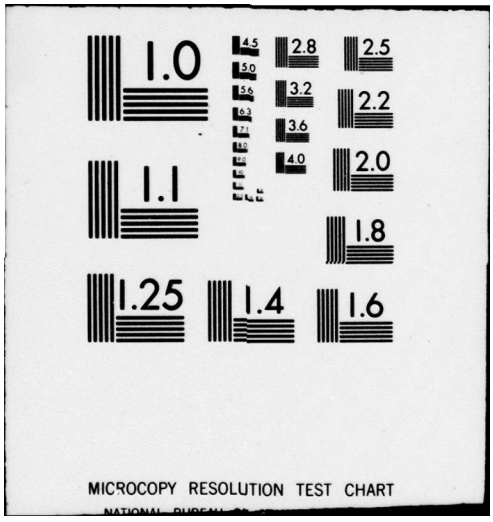
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MECHANISMS OF OXYGEN TOXICITY AT THE CELLULAR LEVEL

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WORK UNIT NO. NR 204-020 CONTRACT: N0014-C-0328

REPORT #32, January 1, 1978

STATUS REPORT, COVERING THE PERIOD SINCE THE ANNUAL REPORT OF JANUARY 1978, AND REQUEST FOR CONTINUATION

This report consists of the following: (I) Summary of Accomplishments, (II) Reseach Remaining to Be Completed this Contract Year, (III) Outline of Future Plans, (IV) Itemized Budget, and Administrative Approval (p. 1).

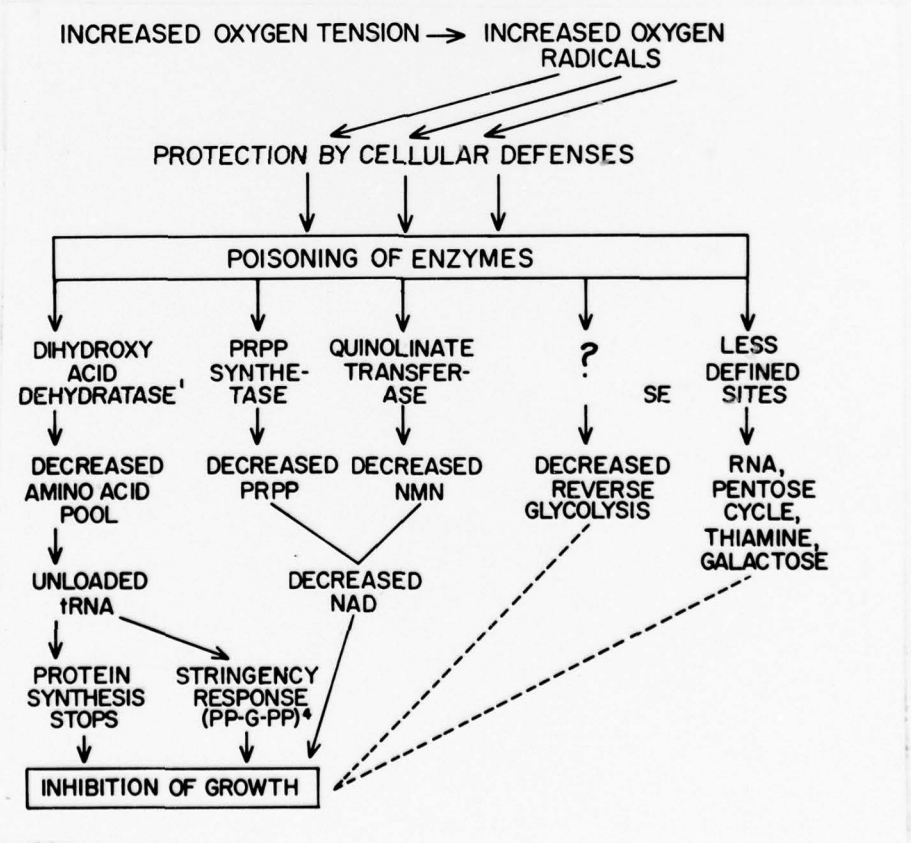
I. Summary of Accomplishments: A summary of significant positive findings during the total contract period will be used to introduce the specific accomplishments of the current contract.

I.a. SUMMARY

Oxygen at elevated pressures is toxic for life forms from microbes to man. Growth is inhibited in Escherichia coli and results primarily from poisoning of specific enzymes in the following biosynthetic pathways: branched-chain and aromatic amino acids, NAD-niacin, phosphoribosylpyrophos-

phate (PRPP) and reverse glycolysis. Inhibition of amino acid biosynthesis indirectly stops protein synthesis and induces "stringency" (production of pp-guanine-pp, a powerful inhibitor of metabolic processes) which accounts for inhibition in metabolic processes where there is no observed enzymatic poisoning. Inhibitions in the PRPP, NAD-niacin, and reverse glycolysis pathways are significant to bacterial (and perhaps to human) oxygen toxicity where products of the pathways protect. These relationships are shown in the following diagram (figure 1).

FIGURE 1. BASIC SITES AND INTERRELATED CONSEQUENSES OF OXYGEN POISONING AT THE CELLULAR LEVEL



I.b. Research completed, as of December 31, 1978, toward specific objectives detailed in the proposal for the period April 1, 1978 - March 31, 1979.

(1) Relationship between guanine polyphosphate (ppGpp) concentration, oxygen inhibition of metaoblism, and the known inhibition of amino acid biosynthesis (part 1-b-i of the proposal).

The ppGpp has been measured in a stringent and in a relaxed strain of E. coli prior to and during oxygen poisoning. Our hypothesis that oxygen poisoning would induce the stringent response, was confirmed. ppGpp increased in concentration in stringent but not in relaxed strains (Figure 2). This supports our previous findings for poisoning of specific enzymes of amino acid biosynthesis which would lead to amino acid starvation and induction of ppGpp production with consequent shut-down of various metaoblic pathways.

We have carried the study further and obtained results beyond those proposed. Specifically, we hypothesized that strigent strains should benefit from the rapid and uniform shut-down of metabolism via rapid ppGpp production during the early exposure to hyperoxia. (By analogy, the stringent response is thought to be of adaptive survival value during periods of deprivation of environmental sources of amino acids for amino acid requiring strains). We have determined that strains which are isogenic except for the stringency gene, do indeed differ in their resistance to oxygen poisoning. The stringent strain, like most wild-type strains, survived 4.2 atm of oxygen with little or no killing for 21 hrs, while the relaxed strain (which does not produce additional ppGpp in response to intracellular dearth of amino acid) showed measureable decrease in viability. The recovery response following removal from hyperoxia was

very different for the two strains. Inhibition of growth was reversed in the stringent strain much more rapidly than in the relaxed strain. Two conclusions regarding the biological role of stringency with respect to survival during the stress of oxygen toxicity are apparent. For a stringent strain, compared to a relaxed strain: (1) more cells would survive and, (2) growth would occur much sooner upon removal from the elevated oxygen state. In direct competition, the relaxed strain would be selected against so that it probably would not survive. The data are shown in Figure 3.

(2) Further evaluation of effects of oxygen on the NAD-niacin pathway (parts 2-b-i through iii):

The proposed research has not been completed. A paper, summerizing progress to date, has been submitted to Biochimica et Biophysica acta. A copy is enclosed as an addendum I. In addition, significant progress has been made in closely correlated research which was not specifically proposed. This research was made possible by the addition of Dr. Foudin to our research group, at no cost to ONR.

Of basic importance is the determination of the extent to which discoveries made with the bacterial sites of oxygen toxicity may apply to cells of higher life forms. To this end, assays of PRPP synthetase activity in erythrocytes, in mouse fibroblast and in Hela cells have been made.

Exposure of whole human blood to hyperbaric oxygen for 30 min. resulted in only a small reduction (about 12%) in PRPP synthetase specific activity (Table 1). Of striking interest, however, is the finding that the PRPP content in the erythrocytes was greatly increased during the period of oxygen exposure. This indicates that biosynthetic

reactions which utilize PRPP are greatly impaired. The poisoning of quinolinate transferase could well account for this, and its activity will be measured.

The data for Hela and mouse fibroblast cells does not reveal any significant impairment of PRPP synthetase during oxygen exposure (Table 2). The PRPP content prior to and following hyperbaric oxygen exposure has not yet been determined in these cells.

It was anticipated that PRPP synthetase specific activity would be significantly reduced in all these cells (Tables 1 and 2) by incubation in hyperoxia. The contrary finding is of interest and may be eventually explained by one of the following: (a) we are in error with respect to the data from either the bacteria or the mammalian cells, (b) the mammalian cells are more resistant due to protective levels of enzymes such as superoxide dismutase, or (c) toxic levels of oxygen radicals were not generated under the conditions of the experiment. We do not feel that (a) is a likely explanation. The resistance of erythrocytes may result from a combination of high concentrations of superoxide dismutase and other protective factors and a low production of oxygen radical species. This would be in keeping with the known function of the red cell as a carrier of oxygen. The resistance of Hela and mouse fibroblasts is more unexpected. The cultures were mature and may actually have been non-proliferating. The actual metabolic rates (including oxygen uptake and subsequent production of radicals) may have been low. Further experiments with young cultures and actual determinations of metabolic rates in hyperoxia are planned. If these data patterns hold, it indicates that poisoning of PRPP synthetase may not be significant to oxygen toxicity in mammals.

The previously mentioned (Table 1) increase in PRPP concentration in erythrocytes after incubation in hyperoxia is most interesting. It possibly is related to our previous findings that: (a) quinolinate transferase (which requires PRPP as a substrate) is poisoned in hyperoxia, and (b) there is impaired synthesis of nucleotides and RNA (which requires PRPP as substrate).

To date, comparisons of the physical characteristics of the enzymes which we have found to be oxygen sensitive, has not revealed any common properties which are definitely associated with the sensitivity. Compilation from the literature of features of the sensitive enzymes is continuing.

(3) In vivo inactivation of Fructose-1,6-diphosphatase by hyperoxia (parts 7-b-i).

A surprising result was obtained when fructose-1,6-diphosphatase specific activity was compared in extracts of Escherichia coli K-12 prior to and following exposure to hyperoxia. Inactivation of the enzyme was minimal (Table 3).

It may be recalled that our previous data had shown that: (a) fructose-1,6-diphosphate (a substrate in the Krebs cycle) was a much poorer protective agent than was fructose-6-phosphate, fructose, or glucose. This was interpreted as evidence that reverse glycolysis was impaired at the enzyme step catalyzed by fructose,1-6-diphosphatase [refer to report #30, December 29, 1977 for a fuller analysis].

When purified fructose-1,6-diphosphate was exposed in vitro to superoxide anion, there was measurable inactivation, while under similar conditions, another control enzyme was not affected. We ten-

tatively concluded that the block in reverse glycolysis in hyperoxia was due to the oxygen sensitivity of fructose-1,6-diphosphatase, but proposed *in vivo* experiments to seek confirmation.

Results of these experiments, as previously stated (Table 3), necessitated a further evaluation by the technique of measuring protection with various intermediates, which was beyond the experiments proposed in the contract.

Table 4 shows the results of these experiments with K12. As previously determined with E26, glucose was the best protective compound, and fructose-6-phosphate was almost as good. Also, as previously determined, fructose-1,6-diphosphate and pyruvate did not protect as well. However, we went further, after these confirmatory experiments to measure whether the supplemented carbohydrates were actually utilized by the bacteria. The test of utilization was to compare the total growth obtained in the presence and in the absence of the added carbohydrate. A new fact was uncovered here. Among the carbohydrates tested (Table 4) only fructose-1,6-diphosphate failed to increase the total yield of cells. This clearly showed that the failure of fructose-1,6-diphosphate to protect was due to incapability of the cells to transport it, and was not due to poisoning of fructose-1,6-diphosphatase which agrees with the data of Table 3.

To further establish this point, glycerol was tested as a substrate (Table 4). It is taken up by the cells and gives protection comparable to fructose-6-phosphate. Glycerol enters the EMP as glyceric acid-3-phosphate (prior to PEP). Together, these data implicate oxygen impairment of phosphoenolpyruvate carboxykinase and phosphoenol pyruvate

synthetase as the cause of inhibition in reverse glycolysis.

Fructose-1,6-diphosphatase, which was previously suspect, is not the site in vivo, and our preliminary conclusions were in error because fructose-1,6-diphosphate does not enter the cell, presumably because of its highly polar nature due to the two phosphates.

Tests of the effect of hyperoxia on phosphoenolpyruvate carboxykinase and phosphoenolpyruvate synthetase activity in cell extracts, are proposed.

(4) Evaluation of the specific enzymatic site of oxygen poisoning of branched chain amino acids. [This research was done to complete objectives of the proposal for the period April 1, 1977 through March 31, 1978.] The research is described in the attached copy of a manuscript, addendum II.

I.C. Personnel associated with the project and funding sources:

Considerable salary support for research on the objectives of the current ONR contract has been provided by University sources.

(a) Olen R. Brown, Ph.D. Principal Investigator, salary paid by University of Missouri.

(b) Laurie Foudin, Ph.D., Post-doctoral trainee, salary paid by NIH Training Grant for the Dalton Research Center, Sept, 1, 1978 - August 31, 1979.

(c) Patti Gilliland, M.S., Research Specialist, salary paid by ONR contract, Sept. 1, 1978 - March 31, 1979.

(d) Frederick Yein, M.S. Research Specialist, salary paid by ONR contract and the Dalton Research Center, April 1, 1978 - July 1978.

(e) Richard Siether, B.S., Graduate student, stipend paid by Dalton Research Center and Graduate School.

(f) Doris, Song, M.S., temporary research Specialist, salary paid by ONR.

I.d. Publications

(In Press or published since renewal request of 1978).

(a) Brown, O. R., Yein, F., and Boehme, D. Bacterial Sites of Oxygen Toxicity Potentially Common to Red Cells and Erythropoiesis. The Red Cell, Proceedings 4th International Conference on Red Cell Metabolism and Function, Ed. by G.J. Brewer, Alan R. Liss, Pub., pp. 701-714, (1978).

(b) Brown, O. R., Yein, F. Sensitivity to and Site of oxygen Poisoning in Escherichia coli. Fifth International Symposium on Intestinal Microecology, Ed. by D. Hentges and T.D. Lucky. In the Press: American Journal of Clinical Nutrition 32 (1979).

(c) Brown, O. R. and Yein, F. Dihydroxyacid Dehydratase: The Site of Hyperbaric Oxygen Poisoning in Branched-chain Amino Acid Biosynthesis. Biochem. Biophys. Res. Comm., 85:1219-1224 (1978).

(d) Brown, O. R., Boehme, D., and Yein, F. Fructose-1,6-diphosphatase: A Cellular Site of Hyperbaric Oxygen Toxicity. (In the Press, Microbios., 1978).

(e) Brown, O. R., Yein, F., and Boehme, D. Oxygen Poisoning of NAD and Phosphoribosylphosphate Biosynthesis: Proposed Sites of Cellular Oxygen Toxicity. (Submitted to Biochimica Et Biophysica Acta).

(f) Brown, O. R. Specific Enzymatic Sites and Cellular Mechanisms of Oxygen Toxicity, (In the Press: Proceedings of 2nd International Conference on Biochemical and Clinical Aspects of Oxygen. Ed. by W.S. Caughey).

Table 1. Effect of Hyperbaric Oxygen on PRPP Synthetase Activity in Erythrocytes Following Treatment of Whole Blood

Treatment prior to assay*	Enzyme activity** (nmoles PRPP/mg/hr)	Relative PRPP content (dpm/assay)***
Control (no treatment)	53.58±5.04	1302±662
HPN (4.8 atm, 30 min)	52.78±4.55	2670±322****
HPO (4.2 atm, 30 min)	47.48±5.40****	5889±1230****

*Whole blood was incubated at 37°C under the specified conditions. Following treatment, erythrocyte hemolysates were prepared and assayed for enzyme activity. The relative PRPP content are the values for extracts incubated without substrates.

**Averages ± 1 S.D. For 12 determinations for 3 experiments.

***Averages ± 1 S.D. for 9 determinations from 3 experiments.

****$P \geq 0.01$, student's t-test.

Table 2. Effect of Hyperbaric Oxygen on PRPP Synthetase Activity in Mammalian Cells

Treatment prior to assay*	Enzyme Activity (μmoles PRPP/mg/hr)	
	Hela	Mouse Fibroblasts
Control	1.94 (2)**	0.403±0.051 (4)
HPN (2.8 atm, 60 min)	2.71 (2)	0.567±0.041 (4)***
HPO (2.2 atm, 60 min)	2.09 (2)	0.469±0.104 (4)

*Cells were incubated at 37°C under the specified conditions. Following treatment, cell-free extracts were prepared and assayed. With Hela Cells (monolayer), the control was prepared directly without further incubation; with fibroblasts, cell-suspensions from monolayers were prepared prior to treatment, and the control suspension was incubated in air for 60 min.

**Number in parentheses indicates number of determinations; with fibroblasts, two concentrations of extract were used.

***$P \geq 0.01$, student's t-test.

Table 3. Effect of High Pressure Oxygen Exposure on Fructose-1,6-Diphosphatase in Escherichia coli K12[1].

Time of exposure (min)	Specific Activity (U/mg)[2]	
	Air	HPO
10	0.172±.022 (12)	0.234±.035 (12)[3]
60	0.172±.032 (24)	0.145±.030 (23)[4]

[1]Cells were exposed to 4.2 atm partial pressure of oxygen (HPO) for the indicated time at 37°C during exponential growth. The enzyme activities were measured in cell-free extracts by the phosphate liberation method of Pontremoli (Methods Enzymology 9:625-631) with inorganic phosphate determined by a modification of the method of Fiske and Subbarow (J.B.C. 66:375-380).

[2]A unit of fructose 1,6-diphosphatase activity liberates one micromole of inorganic phosphate in one minute at 37°C. The numbers in parentheses represent the number of total assays.

[3]Significantly higher than air control at $p \leq 0.005$.

[4]Significantly lower than air control at $p \leq 0.001$, using Student's t-test.

Table 4. Effect of specific carbohydrates on Growth in hyperoxia of *Escherichia coli* K12.

Supplemented Carbohydrate[1]	Gen. Time (min)		Percentage Protection	#gen/hr	Normalized Ratio[2]
	Air	4.2 atm O_2		4.2 atm O_2	
Glucose[3]	38.2±2.34	50.4±6.31	76.6±8.71	1.21±1.44	1.00
Fructose-6-phosphate[3]	28.9±1.62	53.2±3.42	55.1±4.5	1.15±0.68	0.72
Fructose-1,6-diphosphate[4]	37.7±2.72	250.8±46.7	15.6±4.44	0.248±0.52	0.20
Glycerol[3]	34.8±1.09	64.6±3.38	54.1±4.6	0.936±.05	0.71
Pyruvate[5]	36.7±1.16	99.4±1.31	37.0±1.2	0.608±.008	0.49

[1]Medium contains 20 amino acids.

[2]The quotient obtained by dividing the ratio of the air to hyperbaric oxygen generation times by the value of this ratio with glucose present. This ratio compensates for the differences in growth rates in the various media and is normalized with respect to the protection with glucose present. The value of normalized rations decrease as protection by the supplimented carbohydrate decreases, relative to protection by glucose.

[3]Enters the EMP pathway prior to PEP.

[4]Determined by experiments to not enter the cell.

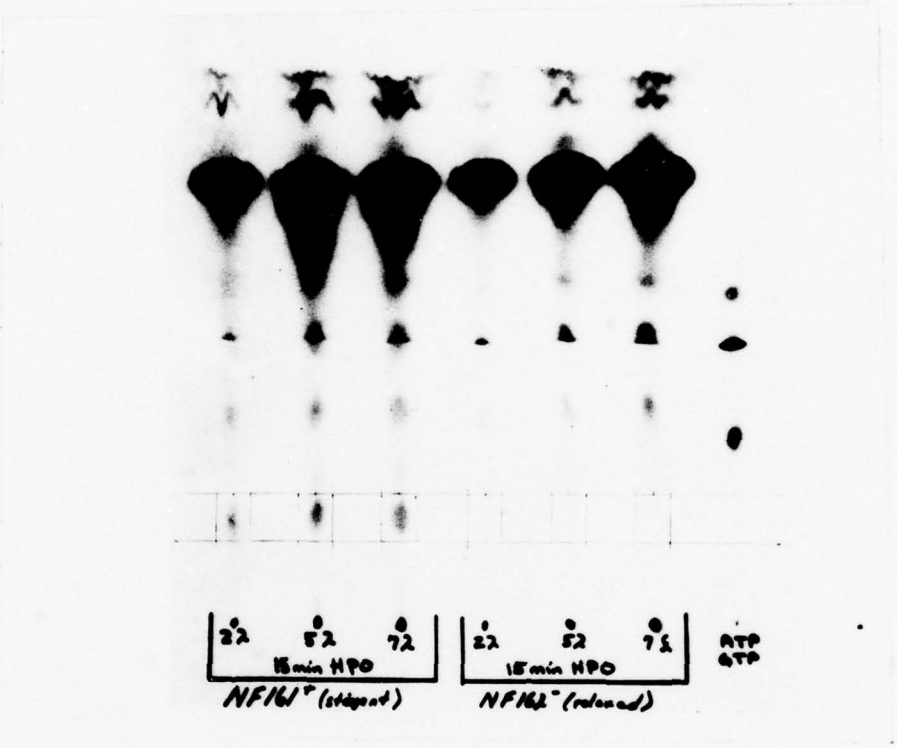
[5]Enters the EMP pathway after PEP.

Figure Legends

Figure 1. Photograph of thin-layer chromatograms of ppGpp produced by stringent and relaxed E. coli strains following exposure to toxic levels of oxygen.

Figure 2. Comparative effects of hyperoxia on stringent and relaxed strains. More cell death (during hyperbaric oxygen exposure) and slower recovery of growth (upon removal from hyperoxia) occurred with the relaxed compared to the stringent strain. The relaxed strain does not produce ppGpp and "unbalance" metabolism continues for a period of time in hyperoxia.

FIGURE 1



The spots appearing in the marked squares on the bottom third of the photograph are ppGpp. They are absent in the relaxed strain but are present in the stringent strain following 15 minutes in 4.2 atm of oxygen.

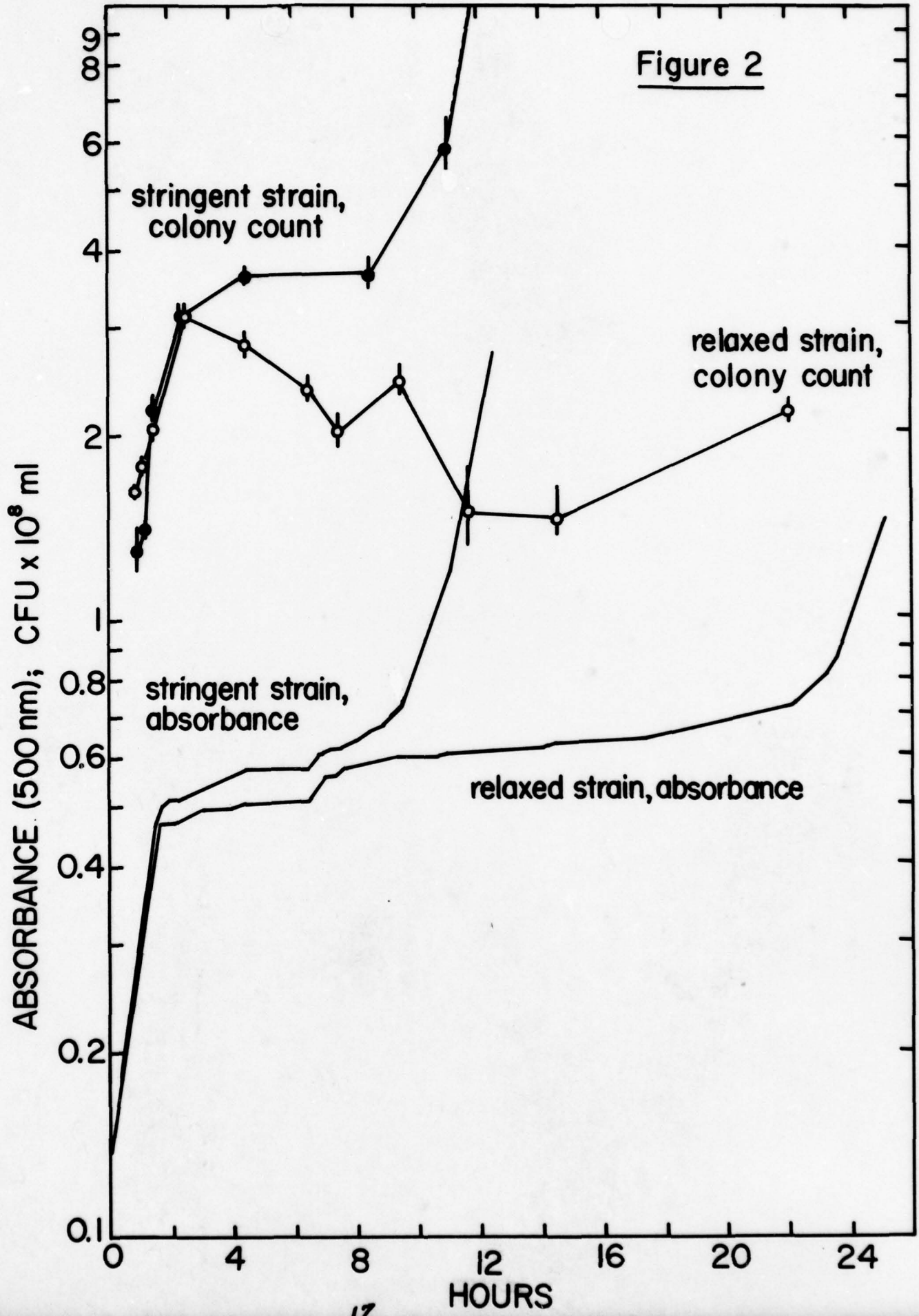


Figure 2
stringent strain, colony count
relaxed strain, colony count
stringent strain, absorbance
relaxed strain, absorbance
ABSORBANCE (500 nm); CFU x 10⁸ ml
HOURS
9
8
6
4
2
1
0.8
0.6
0.4
0.2
0.1
0
4
8
12
16
20
24

ADDENDUM I

OXYGEN POISONING OF NAD AND PHOSPHORIBOSYLPYROPHOSPHATE BIOSYNTHESIS: PROPOSED SITES OF CELLULAR OXYGEN TOXICITY

SUMMARY

Quinolinate phosphoribosyl transferase and phosphoribosylpyrophosphate synthetase, were rapidly inactivated in Escherichia coli exposed to hyperbaric oxygen. The enzymes are essential for biosynthesis of NAD and nucleotides in E. coli and man. Because of their extreme sensitivity and essentiality, inactivation of these enzymes is proposed as a significant mechanism of cellular oxygen toxicity. Niacin and thiamine provided significant protection against growth inhibition from hyperoxia in E. coli and could be useful in cases of human oxygen poisoning.

INTRODUCTION

Life forms which use oxygen must avert the potentially harmful effects of oxygen radicals which can be formed by univalent reduction. The toxic radicals can be converted to harmless species by cellular enzymes such as superoxide dismutases, catalases and peroxidases. However, the margin of safety is narrow and, in general, aerobic life cannot survive in oxygen tensions 5 to 10 times that of air, and anaerobes are very much more sensitive [1]. The universality of the susceptibility of life to oxygen poisoning suggests that specific structures or compounds common to and essential for life, are readily damaged by such radicals. Although significant progress has been made, identification of the specific cellular biochemical sites and mechanisms responsible for the acute central nervous system and chronic lung toxicity of high oxygen tensions has not been achieved [2].

An understanding of oxygen toxicity has basic relevance for understanding of cellular respiration, and applications for therapy. For example, oxygen therapy is valuable in a variety of clinical conditions including gas gangrene, prematurity in infants, carbon monoxide poisoning, and cardiovascular and lung dysfunctions.

Recently, using the facultative bacterium Escherichia coli grown in minimal defined medium, partial protection was found against oxygen toxicity by adding specific amino acids [3]. Certain intermediates of the biosynthetic pathways for these amino acids also protected, indicating that specific enzymes for synthesis of branched-chain and aromatic amino acids were inactivated rapidly upon exposure of Escherichia

coli to hyperbaric oxygen [3]. These data are sufficient to account for the acute growth-cessation in minimal medium as the consequence of inhibition of protein synthesis through deprivation of specific amino acids. However, the protection afforded by these specific amino acids decreased with length of incubation of bacteria in high oxygen tension [3]. This might be expected if oxygen-inhibition of synthesis also occurred for other essential components which are consumed more slowly during growth than are amino acids.

In this paper we report evidence that niacin and thiamine complement the protection against oxygen toxicity provided by the amino acids for Escherichia coli. In hyperbaric oxygen these vitamins apparently cannot be synthesized in the quantities required for growth, consequently there is a deficiency in synthesis of the co-enzymes thiamine pyrophosphate, NAD, and NADP. The mechanism of protection by thiamine has not been investigated further. The specific sites of oxygen inhibition in the NAD-niacin pathway are the enzymes quinolinate phosphoribosyltransferase and 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase. The inability to adequately synthesize PRPP may impede other pathways in which it is a substrate, including nucleic acid synthesis. Since the oxygen-sensitive enzymes are essential also to the functioning of mammalian cells, it appears that the findings are relevant to human oxygen toxicity.

MATERIALS AND METHODS

Experimental conditions for measurement of growth of bacteria in hyperoxia in medium supplemented with various intermediates were as previously described [3]. To measure the effects of hyperoxia on

specific enzymes, Escherichia coli (strain K-12, W3899N) was obtained from Gholson [4], and grown at 37°C in minimal medium [3] containing 27.7 M glucose, 10 mM quinolinate and with or without 20 amino acids [3] (0.65 mM each). Control cultures were grown with a gas phase of air. Cultures in exponential growth was exposed to a gas phase of 1 atm of air plus 4 atm of oxygen (4.2 atm O_2). Where indicated, cultures were reincubated with air as the gas phase to test recovery. To prepare enzyme extracts, cultures were rapidly decompressed and poured into a vessel containing potassium phosphate buffer, 0.05 M, pH 7.3, crushed ice and chloramphenicol at a final concentration of 150 µg/ml. Cells were concentrated by centrifugation and approximately 0.7 g wet weight of cells was disrupted in 3 to 4 ml of same buffer with chloramphenicol at 4°C for 3 min with a Bronwill sonic probe at maximum power setting (30 sec alternating power on and power off). The extract was centrifuged at 4°C for 20 min at 12,000 x g and the supernatant was assayed or further treated with ammonuim sulfate as described by Gholson et al. [5]. The enzyme was assayed as described previously [4] using quinolinate -2,3,7,8-(^{14}C) synthesized as described by Gholson et al. [5]. One unit of enzyme converts 1 µ mole/min of quinolinate into NMN and $^{14}CO_2$. The $^{14}CO_2$ was trapped and measured by liquid scintillation spectrometry.

RESULTS

Five vitamins and parahydroxybenzoic acid (PHBA) were tested for their ability to protect Escherichia coli from the toxic effects of 4.2 atm of oxygen (Table I). These supplements were added to basal

salts medium with glucose and twenty amino acids, a medium previously reported to give significant protection for approximately one generation [3]. Where growth rates with air as the gas phase in the presence of different nutrients were similar, it is reasonable directly to compare generation times obtained in these various media in hyperbaric oxygen (Table I). Where growth rates in air were significantly influenced by addition or deletion of a specific nutrient, the data are better compared after normalization (Table I). With niacin, PHBA, thiamine, biotin, pantothenate, and folate all present, cells grew considerably more (completed more generations) in 4.2 atm of oxygen than did cells in medium without these compounds (Table I). This was evident during the first 2 h of incubation (Table I) in hyperoxia, and the difference was increased on further incubation (2-4 h interval). The inhibition of growth was caused by elevated oxygen tension and not by pressure, per se, since mixtures of air plus helium or nitrogen at the same total pressure did not inhibit. Deletion of these compounds singly and in combinations indicated that only thiamine and niacin were protective, as evidenced by significantly decreased growth ($p \geq 0.05$) upon their deletion, but that PHBA, biotin, acid and folate were not protective (Table I). When valine was omitted as a control (Table i) results were similar to those previously reported [3].

Additional vitamins, bases, and other metabolites which were tested, failed to increase the protection when they were added to medium containing the protective amino acids [3] plus the protective vitamins thiamine and niacin (Table I). To minimize the possibility that failure of a compound to protect against oxygen toxicity resulted from failure of transport, cells were grown for two sub-

cultures in medium containing the test compound to preadapt them. The best protective medium (containing amino acids, niacin and thiamine) gave significant growth in hyperoxia (Fig. 1) compared to basal medium alone or such medium supplemented with the amino acids found to be protective. In the protective medium at 4 h, growth was continuing with a constant generation time of approximately 104 min which was established after 2.5 h of hyperbaric oxygen; without vitamins, essentially no growth was obtained after 70 min in hyperbaric oxygen (Fig. 1). The culture continued to grow (not shown in Fig. 1) and reached an absorbance of 8.0 which was comparable to that obtained by the control with air as the gas phase (Fig. 1). The reduced rate of growth in the control culture after an absorbance of 3 (Fig. 1) was associated with approach of the maximum population density characteristic of the medium. The protection afforded by amino acids, thiamine and niacin was sufficient to indicate that damage by hyperoxia to other systems must be comparatively very much less significant to the cell.

The protective effect of niacin was investigated further. Intermediates of the biosynthetic pathway of nicotinamide adenine dinucleotide (Fig. 2) were tested for their ability to protect E. coli from the growth-inhibiting effects of 4.2 atm of oxygen by adding them to basal salts medium containing glucose and amino acids [3]. Niacin contributes to biosynthesis of NAD and subsequently to NADP. NAD synthesis (Fig. 2) occurs by the same pathway in many life forms (procaryotes and eucaryotes) from the intermediate quinolinate onward [6,7]. Growth was enhanced by each of the intermediates: nicotinate mononucleotide, NAD, niacinamide, and niacin but not by quinolinate (Table II). The increased growth rate with the intermediates was

apparent after 1 h and increased further over the interval 2-4 h with all strains (Table II). PRPP (5-phosphoribysyl-1-pyrophosphate), when added with quinolinate had little, if any measurable effect in this test (Table II). All organisms were preadapted by growth in medium containing the test compounds. However, PRPP is unstable and degrades readily.

The critical steps were confirmed using two mutant strains (W3899 and W3899-N) which are known to transport quinolinate since they grow with quinolinate substituted for niacin [4]. Strain W3899 carries the NAD B locus [4] and will grow with nicotinic acid, nicotinamide, NAD and NMN as a source of the pyridine ring. Strain W3899-N carries the nicotinamide deamidase mutation as well as the NAD B locus and will grow with nicotinic acid, NMN or NAD, but not with nicotinamide [4]. NAD is not used, per se, but enters via the salvage pathway after partial degradation [6].

The simplest interpretation of the increased growth rate achieved by inclusion of intermediates beyond quinolinate in the pathway of NAD biosynthesis (Fig. 2) is that exposure of cells to hyperbaric oxygen blocked the synthesis of PRPP and/or nicotinate mononucleotide. NAD synthesis occurs via quinolinate phosphoribosyl transferase and phosphoribosylpyrophosphate synthetase from quinolinate and PRPP, or from niacin and PRPP by the salvage pathway (Fig. 2).

To find the potential enzymatic site of oxygen poisoning, both the activities of quinolinate transferase [5] and PRPP synthetase [8,9] were measured in extracts prepared from control cells grown with air as the gas phase and from cells after exposure to growth-inhibiting concentrations of oxygen. Table III shows the effect on

quinolinate tranferase of exposure of exponentially growing E. coli to 4.2 atm of oxygen. The inhibitory effect of hyperbaric oxygen for phosphoribosylpyrophosphate synthetase is shown in Table IV. Inactivation was compared using cells from cultures exposed to hyperoxia with and without amino acids and using washed (resting) cells without glucose to test the concept that active metabolism and oxygen utilization was required for damage to occur. Presumably, it is free-radicals of oxygen [1] produced during metabolism, which are detrimental and not molecular oxygen, per se. The data (Tables III, IV) are consistent with this conclusion, since in the absence of glucose no inactivation of enzyme occurred. Indeed, extracts from resting cells had higher specific activity which suggests that some enzyme inactivation may be occurring in cells metabolizing with air as the gas phase. Without amino acids present in the medium, growth in oxygen was greatly reduced (Fig. 1), and unpublished results have shown that oxygen uptake is sharply curtailed almost immediately upon exposure to hyperoxia. With amino acids furnished, growth and respiration continued longer and more enzyme inactivation occurred (Table III).

Cells recover from oxygen toxicity if returned to incubation with air as the gas phase [10]. PRPP synthetase activity was restored to 80% of the activity found in non oxygen-poisoned cells within 10 min after reincubation with air as the gas phase, but recovery occurred to only 15% of control when chloramphenicol was present to prevent protein synthesis during the recovery phase. (Table IV). This indicates that protein synthesis is necessary for recovery of enzyme activity and suggests that the oxygen-inactivation is due to more than a simple reversible oxidation of essential sulfhydryl groups

of the enzyme. This is substantiated by the fact that a reducing agent, dithiothreitol was present in the assay mixtures and did not result in reactivation of the enzyme during the 20 min of the assay.

DISCUSSION

The significance of imparied synthesis of thiamine relates, in part, to the essential role of this compound in its coenzyme form for transketolase reactions in the pentose shunt. Without thiamine pyrophosphate, production of reduced NADP (required for various reductive steps in biosynthesis) and pentoses (required for RNA and DNA synthesis) by the pentose shunt would be curtailed. Thiamine pyrophosphate is also essential for synthesis of branched-chain amino acids and for the decarboxylation-dehydrogenation reactions of pyruvate to acetyl-CoA, and α-ketoglutarate to succinyl-CoA. It is relevant to recall that the latter two metabolic steps were described in 1963 as sites of oxygen toxicity, but with the interpretation that thiamine pyrophosphate was not involved [11]. It is known that thiamine can be oxidized to thiochrome and, therefore, radical species generated during oxygen intoxication may cause intracellular destruction of thiamine. However, measurement of the ultraviolet absorption spectra of thiamine and niacin revealed that in stirred solutions, neither was altered by exposure to molecular oxygen at 4.2 atm for 1.5 h at 37°C.

Oxygen poisoning of quinolinate phosphoribosyltransferase and of PRPP synthetase would have serious consequences for E. coli. Because the biosynthesis pathway of NAD beyond quinolinate (Fig. 2) is the same in man and other mammals as in E. coli [6,7], the findings appear to have general relevance to oxygen toxicity. Without these enzymes NAD

synthesis would cease when the supply of intermediates, including niacin from the salvage pathway (Fig. 2), were depleted. NAD and NADP are coenzymes for over two hundred biochemical reactions and, it has been shown recently that NAD is consumed by major cellular reactions including cleavage of NAD to nicotinamide with formation of polyadenine diphosphoribose in eucaryotes [12-15] and cleavage to form AMP and NMN by bacterial ligases [16,17]. The half-lives of NAD are reported to be approximately 2 h in E. coli and in animals [18-20], and approximately 1 h in cultured human cells [21,22]. Approximately 95% of the synthesized NAD is consumed by catabolic reactions in the nucleus of mammalian cells and only 5% maintains the NAD pool [21]. Blockage of NAD synthesis by hyperoxia, due to the fact that NAD is consumed and has a comparatively short half-life, would result in proportionately rapid onset of metabolic defects. In fact, one of us (Brown) reported that NAD concentration in E. coli decreased during hyperbaric oxygen exposure [23], but the cause was unknown.

The oxygen sensitivity of PRPP synthetase was somewhat surprising since addition of PRPP to the medium did not protect, and growth continued, although at a reduced rate, in hyperoxia in the presence of niacin or niacinamide. PRPP is necessary for utilization of these compounds for NAD synthesis (Fig. 2). Therefore, some PRPP was available in cells in hyperoxia although PRPP synthetase was severely affected (Table IV). Perhaps there are sources of PRPP, other than synthesis by the oxygen-sensitive PRPP synthetase. Alternatively, in cells undergoing oxygen poisoning, PRPP synthetase may be continually synthesized and inactivated yet function sufficiently to provide some

PRPP. Indeed, the very rapid restoration of PRPP synthetase activity on removal of cells to incubation with air as the gas phase supports the conclusion that machinery for enzyme synthesis is minimally damaged in hyperoxia. The failure of added PRPP to protect was probably due to destruction of the compound which is acid and temperature sensitive, or lack of transport by the cells.

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Table I

PROTECTION AGAINST OXYGEN TOXICITY BY NIACIN AND THIAMINE

Medium A contained basal salts, 27.7 MM glucose and twenty amino acids (each at 0.65 mM) as previously described [3]. Medium B was composed of medium A plus thiamine, biotin, pantothenate and folate, each at 0.1 mM and PHBA and niacin, 0.2 mM. Compounds were deteted or supplemented (each at 0.1 mM except bases, 0.5 mM), as indicated

Nutritional Status	Generation time[a] (min) in			Generations Completed		Normalized Growth Rate[b]	
	1 atm air	4.2 atm oxygen		in 4.2 atm oxygen		in 4.2 atm oxygen	
	--	0-2 h	2-4 h	0-2 h	2-4 h	0-2 h	2-4 h
Medium A	30±1.2	97±7.0[c]	4797±1781[c]	1.24[c]	0.03[c]	0.58[c]	0.03[c]
Medium B	25±0.4	47±1.3	112±3.2	2.55	1.07	1.00	1.00
Medium B							
Minus Niacin	27±0.6	77±5.0[c]	803±188[c]	1.56[c]	0.15[c]	0.66[c]	0.15[c]
Minus Thiamine	27±0.5	63±0.3[c]	162±3.6[c]	1.90[c]	0.74[c]	0.81[c]	0.75[c]
Minus PHBA	27±0.6	52±1.5	--	2.31	--	0.97	--
Minus Biotin	27±0.7	49±0.9	97±1.8	2.45	1.24	1.04	1.25
Minus Pantothenate	27±0.6	49±0.7	105±2.7	2.45	1.14	1.04	1.15
Minus Folate	27±0.5	51±0.7	104±2.5	2.35	1.15	0.99	1.16
Minus PHBA, biotin pantothenate, folate	27±0.6	52±0.7	114±6.2	2.31	1.05	0.97	1.06
Minus Thiamine, niacin	26±0.3	97±1.0[c]	1030±180[c]	1.24[c]	0.12[c]	0.50[c]	0.11[c]
Minus Valine	26±1.3	1242±215[c]	2884±352[c]	0.10[c]	0.04[c]	0.04[c]	0.04[c]

Table 1 (Continued)

Nutritional Status	Generation time[a] (min) in			Generations Completed		Normalized Growth Rate[b]	
	1 atm air	4.2 atm oxygen		in 4.2 atm oxygen		in 4.2 atm oxygen	
	--	0-2 h	2-4 hr	0-2 h	2-4 h	0-2 h	2-4 h
Medium B							
Plus pyridoxine	25±1.6	50±0.6	98±2.7	2.40	1.22	0.94	1.14
Plus Riboflavin	27±0.3	49±0.8	104±5.4	2.45	1.15	1.04	1.15
Plus PABA	26±0.4	52±0.9	103±3.9	2.31	1.17	0.94	1.13
Plus α-Ketioso-valerate	25±0.7	52±0.7	114±2.4	2.31	1.05	0.90	0.98
Plus adenine, thymine, guanine, cytosine, uracil	24±0.4	51±0.3	144±4.8	2.35	0.83	0.88	0.75

Footnotes to Table 1

[a]Averages ± S.E.M. for 8 or more replicate cultures. Generation time (min) = Δt (min) ÷ 3.3 log (A_f/A_i) where Δt = final time - initial time, A_f and A_i = absorbance at 500 nm wavelength at final and initial time, respectively. Generation time becomes infinite as growth rate (Δ absorbance) decreases to zero; hence, the large values and standard deviations as growth decreased. For example, with valine omitted, the average change in absorbance over the final 2 h in hyperoxia was only 0.007 absorbance. Cultures (3 ml) were transferred from exponentially growing cultures and were grown with stirring in vials at 37°C with air as the gas phase for 30 min and the growth rate was determined. The cultures were pressurized, generally at an absorbance of 0.2 to 0.3. with 1 atm of air plus 4 atm of oxygen (4.2 atm total oxygen pressure). The observed growth inhibition was due to hyperoxia and not pressure, per se, since growth rate was not reduced in 1 atm of air plus 4 atm of helium or nitrogen (not shown).

[b]Normalized growth rate = a/b ÷ c/d where the generation times (min) in air in specific test medium = a; in hyperoxia in specific test medium = b; in air in control medium = c; in hyperoxia in control medium = d. Control medium was medium A. A ratio of 1.00 is obtained when the nutrient deletion or supplementation has no effect; ratios significantly less than 1.00 (niacin, thiamine) with nutrients deleted, and greater than 1.00 with nutrients supplemented (none occurred) indicates protection against hyperoxia by the nutrient.

Table II

INHIBITION OF NAD BIOSYNTHESIS BY OXYGEN

The growth and analysis conditions and the basal medium were the same as described for Table I medium A, except that the indicated coumpouds (0.1 mM) were substituted for niacin. NMN is nicotinamide mononucleotide, NAD is nicotinamide adenine dinucleotide, and PRPP is 5-phosphoribosyl-1-pyrophosphate.

Compound Added	*E. Coli* Strain[b]	Generation time (min)[a]			Generations Completed in 4.2 atm oxygen		
		1 atm air	4.2 atm oxygen				
		--	0-2 h	2-4 h	0-2 h	2-4 h	0-4 h
Quinolinate	E-26	26±0.8	80±2.7	383±26	1.50	0.31	1.81
NMN	E-26	27±0.4	52±0.4[c]	190±12[c]	2.31[c]	0.63[c]	2.94[c]
NAD	E-26	29±0.8	56±0.4[c]	167± 4[c]	2.14[c]	0.72[c]	2.86[c]
Niacinamide	E-26	26±0.4	53±0.4[c]	128± 2[c]	2.26[c]	0.94[c]	3.20[c]
Niacin	E-26	25±0.4	47±1.3[c]	112± 3[c]	2.55[c]	1.07[c]	3.62[c]
None	E-26	27±0.6	77±5.0	803±188[c]	1.56	0.15	1.71
Quinolinate	K-12(W3899)	35±0.6	85±1.0	241± 3	1.41	0.50	1.91
Niacin	K-12(W3899)	36±0.9	56±1.7[c]	119± 4[c]	2.14[c]	1.01[c]	3.15[c]
Quinolinate	K-12(W3899-N)	35±0.7	78±0.9	262±17	1.54	0.46	2.00
Niacin	K-12(W3899-N)	33±0.5	52±0.6[c]	118± 2[c]	2.31[c]	1.02[c]	3.33[c]
Quinolinate plus PRPP	K-12(W3899-N)	34±0.1	94±2.2	180± 4[c]	1.28	0.67	1.95

[a]Averages ± S.E.M. for 8 or more replicates anaylzed as described for Table I.

[b]Strains sources identified in text.

[c]Significantly different ($p \geq 0.05$) compared to growth for the strain with quinolinate.

Table III

EFFECTS ON QUINOLINATE PHOSPHORIBOSYLTRANSFERASE ACTIVITY OF ONE HOUR EXPOSURE OF ESCHERICHIA COLI TO HYPERBARIC OXYGEN

Escherichia coli strain K-12 (W3899N) was grown at 37°C in minimal medium containing 27.7 mM glucose, 10 mM quinolinate, both with and without the 20 amino acids [3] (0.65 mM each). HPO = 1 atm of air plus 4 atm of oxygen (4.2 atm O_2). The enzyme assay procedure and definition of a unit of activity are given in Materials and Methods. Averages ± S.E.M. are shown with the number of experiments and total determinations in parenthesis

Gas Phase	Medium	Specific Activity (μU/mg)	
		Crude extract	$(NH_4)_2SO_4$ fraction
Air	Minimal, without amino acids	41.9 ± 2.11(5,20)	43.1 ± 3.43(2,8)
HPO	Minimal, without amino acids	31.5 ± 1.54(2,8)[a]	20.1 ± 1.68(2,8)[a]
HPO	Minimal, with amino acids	3.3 ± 0.31(3,8)[a]	3.2 ± 0.23(2,7)[a]

[a]Significantly lower ($p \geq 0.001$) compared to air controls, using Students' t test.

TABLE IV

EFFECT OF HYPERBARIC OXYGEN ON PRPP SYNTHETASE ACTIVITY

Bacteria and growth and exposure conditions were as described in Table III. Where indicated, cells were reincubated with air as the gas phase to test recovery. Enzyme extracts were prepared as described for Table III, except that tris buffer (0.2M pH 7.5) was used.

Exposure Conditions	Specific Activity[a] (nmoles PRPP/mg protein/h)	
Medium without Amino Acids		
Air Control (4,22)	22.8 ± 1.03	(100%)
HPO, 10 min (2,10)	1.5 ± 0.19	(6.6%)[b]
HPO, 30 min (1,6)	1.2 ± 0.17	(5.3%)[b]
HPO, 30 min + Air, 30 min (1,6)	0.21± 0.05	(0.9%)[b]
Medium with Amino Acids		
Air Control (2,8)	27.5 ± 2.99	(100%)
HPO, 10 min (2,8)	0.95 ± 0.17	(3.5%)[b]
HPO, 10 min + Air, 10 min with chloramphenicol[c] (2,8)	4.10 ± 1.35	(14.9%)[b]
HPO, 10 min + Air, 10 min without chloramphenical[c] (2,8)	22.5 ± 3.61	(81.8%)
washed cells in medium without carbon, energy or nitrogen source[d]		
HPO, 10 min (2,8)	31.6 ± 1.21	(138%)[b]

[a]Mean ± 1 S.E.M. with per cent of respective air control shown in parenthesis.

‡Significantly different ($P \geq 0.001$) compared to air control using Student's t test.

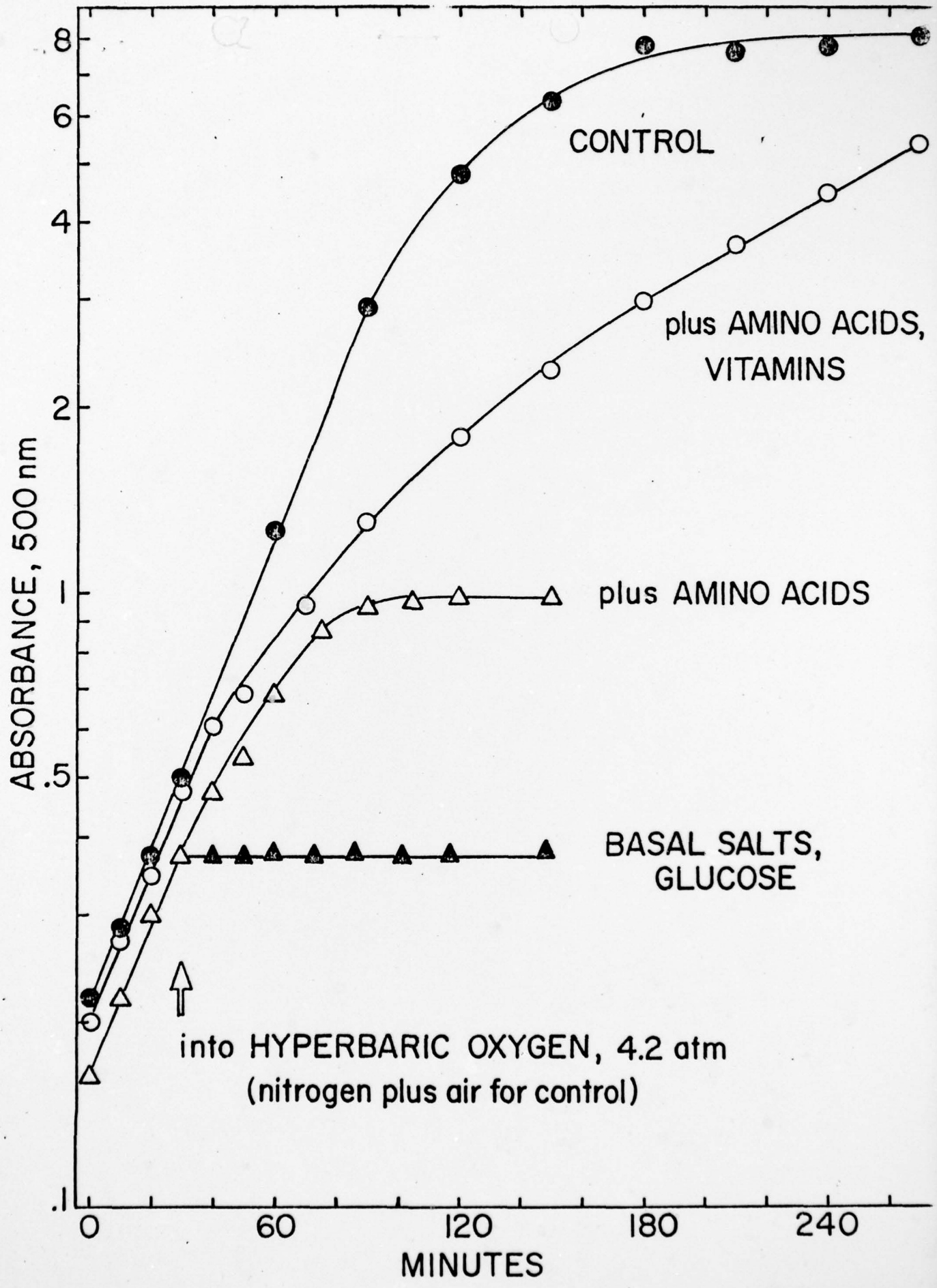
[c]CM = 150 mg/ml of chloramphenicol to inhibit protein synthesis was included during recovery with air as the gas phase.

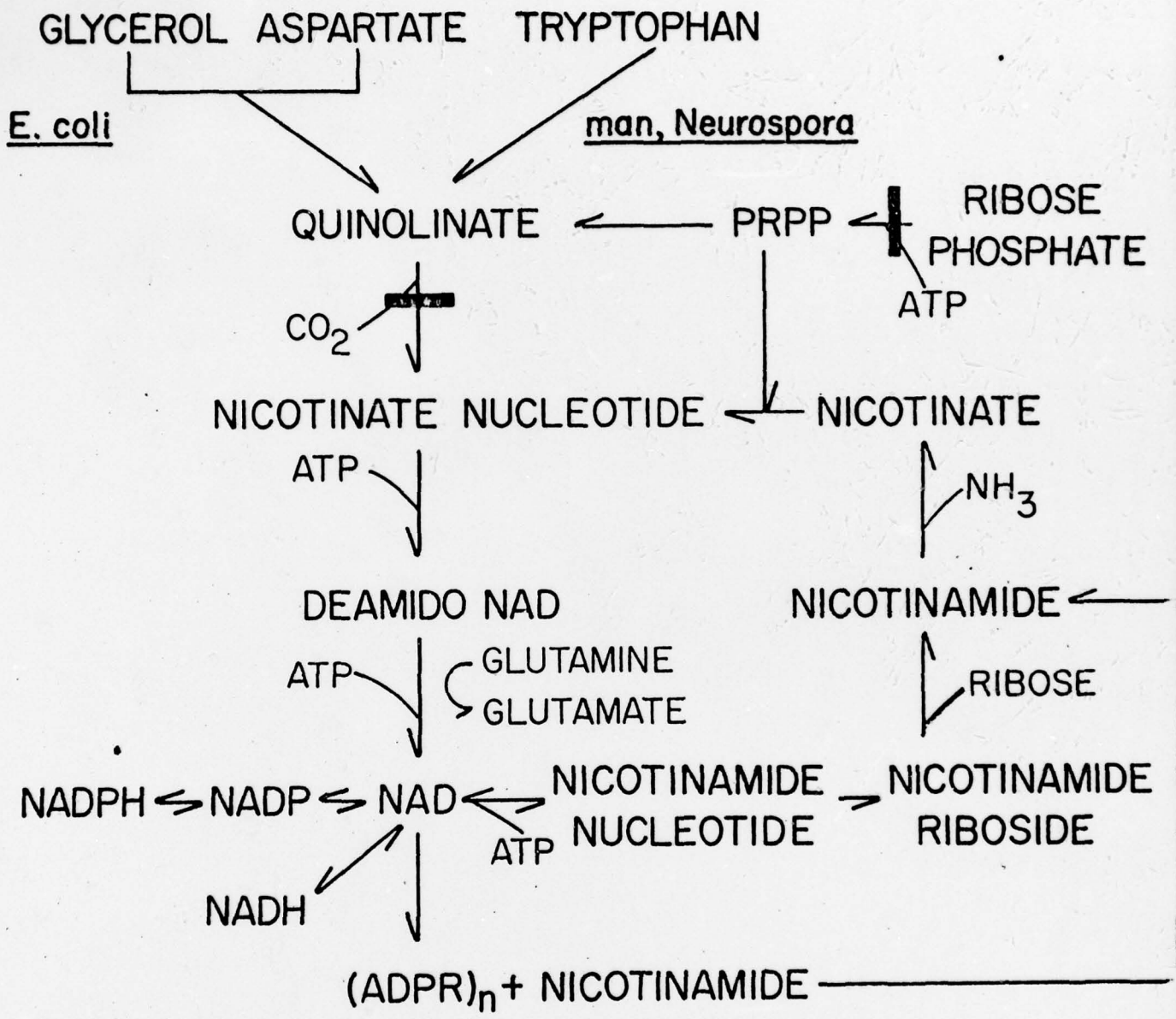
[d]Cells were grown in medium without amino acids and centrifuged and resuspended in basal salts medium [3] without ammonium chloride or glucose and incubated for 10 min at 37°C to produce "starved" cells with low metabolism and oxygen utilization.

Figure Legends

Figure 1. Protective effects of amino acids and vitamins for growth of Escherichia coli in 4.2 atm of oxygen. Strain E-26 was grown in a stirred vessel with air as the gas phase. Cultures were pressurized with 4 atm overpressure of oxygen (gas phase, 1 atm of air plus 4 atm of oxygen) at 30 min in minimal basal salts plus glucose meduim[3] (▲), and the same medium supplemented with 20 amino acids[3], each at 0.65 mM (△), or 20 amino acids plus niacin and parahydroxybenzoic acid (PHBA), each at 0.2 mM, and pantothenate, biotin, folate, and thiamine, each at 0.1 mM (o). Cultures in the medium containing amino acids, vitamins and PHBA were also exposed continuously to air as a control (●). The same results were found (not shown) for the 10 amino acids previously found to protect[3] plus niacin and thiamine as is shown for 20 amino acids plus 5 vitamins and PHBA (o). Data were obtained by removing samples at intervals through a valve without decompressing the parent culture. The absorbances of the samples were measured at 500 nm wavelength with a Gilford spectrophotometer. The relationship between absorbance and plate colony counts is linear up to 0.8 A; all measurements were made below 0.8 A by dilution. There are approximately 3×10^8 cells per ml per 1.0 A.

Figure 2. De novo and salvage pathways for synthesis of NAD as described by Preiss and Hardler[5], and Gholson[4]. The pathways are the same in eukaryotes and in prokaryotes beyond quinolinate.


ABSORBANCE, 500 nm
8
6
4
2
1
.5
.1
CONTROL
plus AMINO ACIDS, VITAMINS
plus AMINO ACIDS
BASAL SALTS, GLUCOSE
into HYPERBARIC OXYGEN, 4.2 atm
(nitrogen plus air for control)
0
60
120
180
240
MINUTES


GLYCEROL ASPARTATE TRYPTOPHAN
E. coli
man, Neurospora
QUINOLINATE
PRPP
RIBOSE PHOSPHATE
ATP
CO_2
NICOTINATE NUCLEOTIDE
NICOTINATE
ATP
NH_3
DEAMIDO NAD
NICOTINAMIDE
ATP
GLUTAMINE
GLUTAMATE
RIBOSE
NADPH
NADP
NAD
NICOTINAMIDE NUCLEOTIDE
NICOTINAMIDE RIBOSIDE
ATP
NADH
$(ADPR)_n$ + NICOTINAMIDE